Ginsenoside Rg1 protects against D-galactose induced fatty liver disease in a mouse model via FOXO1 transcriptional factor

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ABSTRACT

Aims: Rg1 is the most active component of traditional Chinese medicine ginseng, having anti-aging and anti-oxidative stress features in multiple organs. Cellular senescence of hepatocytes is involved in the progression of a wide spectrum of chronic liver diseases. In this study, we investigated the potential benefits and mechanism of action of Rg1 on aging-driven chronic liver diseases.

Materials and methods: A total of 40 male C57BL/6 mice were randomly divided into four groups: control group; Rg1 group; Rg1 + D-gal group; and D-gal group. Blood and liver tissue samples were collected for determination of liver function, biochemical and molecular markers, as well as histopathological investigation.

Key findings: Rg1 played an anti-aging role in reversing D-galactose induced increase in senescence-associated SA-β-gal staining and p53, p21 protein in hepatocytes of mice and sustained mitochondria homeostasis. Meanwhile, Rg1 protected livers from D-galactose caused abnormal elevation of ALT and AST in serum, hepatic steatosis, reduction in hepatic glucose production, hydrogenic degeneration, inflammatory phenomena including senescence-associated secretory phenotype (SASP) IL-1β, IL-6, MCP-1 elevation and lymphocyte infiltration. Furthermore, Rg1 suppressed drastic elevation in FOXO1 phosphorylation resulting in maintaining FOXO1 protein level in the liver after D-galactose treatment, followed by FOXO1 targeted antioxidase SOD and CAT significant up-regulation concurrent with marked decrease in lipid peroxidation marker MDA.

Significance: Rg1 exerts pharmaceutic effects of maintaining FOXO1 activity in liver, which enhances anti-oxidation potential of Rg1 to ameliorate SASP and to inhibit inflammation, also promotes metabolic homeostasis, and thus protects livers from senescence induced fatty liver disease. The study provides a potential therapeutic strategy for alleviating chronic liver pathology.

1. Introduction

The involvement of senescence in liver disorder has drawn increasing attention recently. Amounts of documents using in-vitro and in-vivo experiments from animal models and humans have shown that hepato-cellular senescence is related to the progression of a wide spectrum of chronic liver diseases including viral hepatitis B and C, alcohol-related liver disease, non-alcohol-related fatty liver disease [1]. Taken that chronic liver disease is becoming one of the most serious health problems, whereas Non Alcoholic Fatty Liver Disease (NAFLD) is confirmed as the most common one with its increasing prevalence due to aging, obesity and diabetes, it is increasingly important to understand the underlying mechanism of senescence driving NAFLD and to find effective therapeutic strategy for limiting chronic hepatic disorders [2]. NAFLD is a chronic progressive liver disease which starts with simple hepatic steatosis and progresses to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, even hepatocellular carcinoma [3,4]. Previous studies revealed that high fatty diet (HFD) and high cholesterol diet (HCD) are well-established risks during NAFLD emergence and progression; whereas cellular senescence was always found to be a secondary phenomenon concurrent with the pathogenesis and development of NAFLD/NASH in HFD or HCT treated livers [5], where an enormous burden of senescence was generated as many as 80% of aging hepatocytes in advanced disease [1,6]. A reduction in the number of senescent cells in vivo was accompanied by attenuated steatosis, on contrary, senescence induction in vivo and in vitro promoted hepatic lipid deposition [7]. However, so far, the mechanism of senescence caused hepatic dysfunction remains to be investigated.

The reason for hepato-cellular senescence is focused on oxidative stress. Oxidative stress may cause DNA double-strand breaks (DSB), one of the most severe cellular DNA damage, then generally trigger p53 through ATM dependent phosphorylation, which in turn up-regulates...
p21, a cyclin-dependent kinase inhibitor, to initiate cell cycle arrest [8,9]. It was reported that senescence-associated signal p21 and DSB marker γH2AX increased in mice with HFD induced NAFLD, and more severe steatosis displayed higher level of p21. Moreover, the phenomena are similar to human patients in whom the degree of oxidative stress was also closely related to NAFLD progression and to the DNA methylation of tumor suppressor genes such as p53, p21 and γH2AX [1,6,10,11]. Antioxidant gene knock-out experiment further confirmed the significant role of oxidative stress in NAFLD development. Both senescence marker protein SMP-30-knockout mice on Leprdb/db background as well as SMP30/Superoxide dismutase-1 double-knockout mice developed hepatic steatosis [12,13]. Senescent cells secrete senescence-associated secretory phenotype (SASP), a complex mixture of molecular mediators such as proinflammatory cytokines including IL-1β, IL-6, IL-8, chemokines including Monocyte Chemoattractant Protein-1 (MCP-1), growth factors Fibroblast Growth Factor (FGF), proteases including matrix Metallproteinases (MMPs), concurrent with reactive oxygen species (ROS) and nitric oxide [14,15]. These factors alter the tissue microenvironment as they induce inflammation [16]. Also, SASP may induce senescence in neighboring cells in a paracrine manner. In contrast to apoptosis; senescent cells are stably viable and have pro-tumorigenic properties [17]. Therefore, long-term existed senescent cells are harmful to some extent, and should be removed by therapeutic strategy.

Documents evidenced that metabolic dysregulation, generally referred to glucose and lipids metabolism alteration, may lead to secondary complication such as NAFLD. Meanwhile, metabolic dysregulation is thought to favor cellular senescence in metabolic tissue [18,19]. However, the mechanism between senescence and metabolic imbalance in livers has been uncertain yet. Forkhead box O (FOXO) transcription factors are key downstream regulators in the insulin/insulin-like growth factor 1 signaling pathway, and play a critical role in the regulation of glucose, triglyceride, cholesterol homeostasis [20]. FOXOs maintain hepatic glucose production (HGP). FOXOs transcriptionally regulate gluconeogenesis via activating gluconeogenic genes including glucose-6-phosphatase catalytic subunit [21]. Meanwhile, FOXOs also inhibit glycosylation by suppression of glucokinase and pyruvate kinase gene expression [22,23]. FOXOs suppress denovo lipogenesis. FOXOs may regulate hepatic lipid metabolism via multiple targeted multiple pathways. FOXOs may transcriptionally suppress sterol regulatory element binding protein (SREBP) 1, the lipogenic master regulator, thus inhibit a series of genes involved in fatty acid biosynthesis [24,25]. Moreover, FOXOs activate lipolysis and fatty acid oxidation genes [26]. In addition, FOXOs promote lipid droplet breakdown via activation of lipophagy which is a different pathway from enzymatic lipolysis for lipid degradation [27]. FOXOs also downregulate hepatic cholesterol metabolism via directly inhibition SREBP-2 that targets genes for cholesterol biosynthesis [28]. As playing a critical role in glucose and lipid homeostasis, it is becoming to realize that FOXOs are closely related to NAFLD, nevertheless studies are needed to clarify the role of FOXOs in senescence-induced NAFLD.

D-galactose is widely regarded as an agent for induced senescence. Excessive of D-galactose under the action of galactose oxidase may generate aldohexose and hydrogen peroxide and promote the generation of oxygen-derived free radicals and the superoxide anion [29]. In the current study, D-galactose was used to trigger oxidative stress, thus mimicked progressive cellular senescence in liver as the clinical scenario in chronic liver disease. Ginseng is widely used tradition Chinese medicine and claimed to have anti-aging, anti-oxidative, and anti-inflammatory effects. Ginseng contains multiple active components, including ginsenosides, fatty acids, poly-saccharides, and mineral oils [30], among which the pharmacute effects of ginseng are mainly attributed to ginsenosides, and R1g is representative component with the most active and abundant steroid ginsenosides [31]. Previous literature reported anti-aging and anti-oxidative stress feature of Rg1 in multiple organs [32,33]. Here, we found that Rg1 alleviated hepatocyte senescence, inhibited SASP secretion, suppressed inflammation, protected livers from NAFLD, and the mechanism may be involved in regulation of FOXO1 and targeted antioxidase activity.

2. Materials and methods

2.1. Reagents and antibodies

Rg1 (purity > 95%) was purchased from Hongjiu Biotech Co., Ltd., (Tonghua, China). D-galactose (D-gal; purity > 99%) was obtained from Sangon Biotech Co, Ltd. (Shanghai, China). AST, ALT, SOD, CAT and MDA assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The senescence-associated β-galactosidase (SA-β-gal) staining and colorimetric TUNEL apoptosis assay kits were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The oil red O was purchased from Beijing Solabio Technology Co., Ltd. (Beijing, China). IL-1β, IL-6 and MCP-1 ELISA kits were obtained from Meimian Industrial Co., Ltd. (Jiangsu, China). The antibodies against p53, p21, FOXO1, p-FOXO1 and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibodies against Bcl-2 and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against Bim was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

2.2. Animals and treatments

SPF male C57BL/6J mice aged 6–8 weeks (16 ± 2 g) were supplied by Medical and Laboratory Animal Center of Chongqing (Approval No. SCXK (Yu) 2018-0003; Chongqing, China). All animal experiments were performed in accordance with the institutional and national guidelines and regulations, and approved by the Chongqing Medical University Animal Care and Use Committee. C57BL/6J mice were maintained on a 12 h light/dark cycle at 22 ± 2 °C with ad libitum access to a standard chow diet (n = 40). After one-week acclimation to the condition of housing facilities, the mice were randomly divided into four groups: Control group, Rg1 group, the Rg1 +D-gal group, and D-gal group. In the current study, D-galactose and Rg1 were injected intraperitoneally into mice for 42 d. In the Rg1 +D-gal group, since the 16th day of D-gal injection, ginsenoside Rg1 (40 mg/kg/day) was injected intraperitoneally daily for 27 d concomitantly. In the Rg1 group, ginsenoside Rg1 (40 mg/kg/day) was intraperitoneally injected for 42 d. All control animals were given 10 ml/kg body weight normal saline intraperitoneally.

2.3. Liver index assay

On the 43rd day of the experiment, all mice were weighted and anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 40 mg/kg. The livers were isolated and weighed. Liver index (%) = liver weight / body weight × 100.

2.4. Biochemical analysis

When the mice were killed, blood samples were collected from the retro-orbital plexus (0.3 ml per mice) and centrifuged at 3500 × g for 10 min at 4 °C. ALT and AST in serum were measured using the respective detection kits (JianCheng Biotechnology, Nanjing, China). In addition, liver homogenates were prepared with ice-cold saline (10%, w/v) and centrifuged at 2500 × g for 10 min at 4 °C. Liver MDA, SOD, and CAT activities were determined with the corresponding biochemical kits (JianCheng Biotechnology, Nanjing, China). All the procedures were performed based on the manufacturers’ protocols [34].

2.5. Detection of SA-β-gal activity

Frozen tissues were snap-frozen in liquid nitrogen, embedded in
OCT compound and sectioned into 6 μm slices. Frozen sections warmed to room temperature were fixed in 4% formalin. Subsequently, the sections were incubated with senescence-associated β-galactosidase (SA-β-gal) staining solution (pH = 6) for 24 h. Images of the sections were captured with a microscope and analyzed using the image analysis program Image J.

2.6. Ultrastructure of the liver

Fresh livers were cut into 1 mm3 pieces, immediately fixed within 2.5% glutaraldehyde at 4 °C. The tissue was dehydrated with graded ethanol and embedded within epoxy resin. After polymerization, 70 nm ultrathin sections were made with a diamond knife using Reichert-Nissei ultra-cuts then stained with both uranyl acetate and lead citrate. The stained sections were then observed and photographed using transmission electron microscopy (TEM).

2.7. Histopathological examinations

Completely fixed livers were embedded in paraffin blocks, sectioned at 4 μm, tissue sections were mounted on glass slides and stained with hematoxylin-eosin (HE) to assess the general liver structure. Sections were also stained with periodic acid Schiff (PAS) for measuring changes in glycogen. Apoptotic cells on sections were detected using colorimetric TUNEL apoptosis assay kit. Hepatic morphology was observed with a light microscope.

2.8. Lipid staining

Frozen sections (8 mm) of liver were air-dried for 30 min. The sections were then fixed in 4% formalin for 1 h and washed with tap water. Sections were stained with oil red O mixed with 60% isopropanol for 30 min and then rinsed with 60% isopropanol. Sections were stained with Mayer’s hematoxylin, washed in tap water and covered with aqueous mounting medium.

2.9. Enzyme-linked immunosorbent assay

For detection of IL-1β, IL-6 and MCP-1 in liver homogenates, samples and HRP-conjugated reagent were incubated in the 96-well microtiter plate with for 1 h at 37 °C, followed by chromogen solution at 37 °C for 15 min. Finally, the reaction was terminated by the addition of a sulphuric acid solution and all optical densities were measured at 450 nm. Each sample was designed with three replicates randomly.

2.10. Immunohistochemical assays

Four-micrometer sections were deparaffinized with xylene and rehydrated in ethanol series. The sections were treated with 3% hydrogen peroxide to inactivate the endogenous peroxidase activity and then immersed in citrate buffer at 95 °C for 20 min to retrieve the antigen. After blocking 10% goat serum, tissue sections were incubated with primary antibody against FOXO1 (1:200 dilution) at 4 °C overnight. On the second day, after washing in PBS, the tissues were incubated with a biotinylated anti-rabbit link secondary antibody for 30 min. Sections were incubated with DAB colour substrate and counterstained with Mayer’s hematoxylin. FOXO1 proteins were represented by a brown or yellow granular mass and quantified the amount of immunostaining by ImageJ software.

2.11. Western blot assay

To detect P53, P21, FOXO1, p-FOXO1, Bcl-2, Bax and Bim proteins, the liver tissue homogenates were extracted using ice-cold tissue lysis buffer. Total protein concentration was determined by BCA protein assay kit. Equal amounts of protein (40 μg/lane) were loaded onto 10% SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA). Membranes were blocked with 5% skim milk in TBS with 0.1% Tween-20 for 1 h at room temperature and incubated overnight at 4 °C with a 1:1000 dilution of various antibodies. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:10,000, Southern Biotech, Birmingham, AL, USA) for 1 h at room temperature. Protein expression was detected by an enhanced chemiluminescence (ECL) method and imaged with ChemiDoc XRS (BIO-RAD, Hercules, CA, USA). The intensities of the protein bands were analyzed using Image Lab 5.2.1 (BIO-RAD, Hercules, CA, USA).

2.12. Statistical analysis

All the data were presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 22.0 (IBM Corp, Armonk, NY, USA). Data were analyzed by one-way ANOVA, followed by t-tests. P < 0.05 was considered statistically significant.

3. Results

3.1. Rg1 protected livers against d-galactose induced damage

After 42 days of continuous treatment, liver indexes in the D-galactose (D-gal) group were significantly higher compared with the control due to increased liver weight; however, Rg1 treatment recovered the liver index of induced-senescent mice (Fig. 1A, B). Release of the intracytoplasmic enzymes, mainly ALT and AST, is an indicator of liver injury, therefore serum level of ALT and AST was measured. Administration of d-galactose significantly elevated the biochemicalFig. 1. Rg1 reduced the D-galactose caused damage to the liver. (A) The chemical structure of ginsenoside-Rg1. (B) Liver index was demonstrated by the ratio of the liver weight (g) to the mouse body weight (g). The increase in liver index indicated the hepatotoxicity induced by d-galactose treatment; however, Rg1 restored the liver index (n = 3/group). (C, D) Rg1 reversed liver biochemical parameters ALT and AST abnormal elevation after D-galactose treatment (n = 3/group). Serum levels of ALT and AST were presented respectively. Data are presented as means ± SD (n = 5/group). ***P < 0.01; ****P < 0.001.
Fig. 2. Rg1 attenuated the senescence of hepatocytes induced by α-galactose treatment. (A) Frozen sections of liver tissue were analyzed by senescence-related SA-β-gal staining. Senescent hepatocytes are blue-green stained (magnification 200×). (B) Western blots were performed to determine the expression level of p53 and p21 proteins in liver tissue homogenates. β-actin was probed as a loading control. (C) A histogram of relative protein expression is presented. (D) Rg1 treatment preserved the mitochondria microstructure evaluated by TEM (n = 3/group). Representative liver TEM micrographs showing swelling mitochondria, disintegration of their cristae and loose cellular matrix of hepatocyte in the D-gal group, however well-preserved organelles after treated with Rg1. Scale bar are 500 nm and 1 μm respectively. The arrow points to the lipid droplet; the hollow arrows point to the myelin figures; the double arrows point to the lipofuscin; and the asterisks mark the swelling mitochondria. The autophagosome is shown within the box at a higher magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
parameters compared with the control group; however, treatment with Rg1 significantly decreased the abnormal serum level of ALT and AST compared with the α-galactose group (Fig. 1C, D). These results indicated that α-galactose generating oxygen-derived free radicals and the superoxide anion impaired the function of hepatocytes, however, Rg1 protected the liver against the damage.

3.2. Rg1 protected livers against α-galactose induced senescence

SA-β-gal is a classical biomarker particular for senescent cells. As shown in Fig. 2A, frozen sections of the liver from α-gal group demonstrated a widespread and diffused SA-β-gal positive staining. After treatment with Rg1, the sections of the α-gal + Rg1 group revealed a sporadic distribution of SA-β-gal positive cells. Tumor suppressor P53 and its downstream transcriptional target P21 are pivotal signaling for early senescence. Western Blot results demonstrated significant increases in both P53 and P21 in α-galactose treated hepatocytes, whereas decreases in the Rg1 singly treated group. Furthermore, Rg1 post-treatment reversed the abnormal increase of P53 and P21 in the α-galactose treated livers (Fig. 2B, C). Mitochondria are considered as the aging clocks which are the main targets of free radical attack; meanwhile damage of mtDNA may be the reason for cellular aging. In the current study, TEM examination of liver tissues revealed swelling mitochondria, cavitations in the mitochondrial cristae, and a loose hepatocellular matrix in the α-gal group. However, in the Rg1 + α-gal group, mitochondrial swelling and vacuolation were alleviated and cytoplasmic matrix was more intact than that in α-gal group (Fig. 2D). The results above suggested that α-galactose successfully led to senescent signs of hepatocytes; notably, Rg1 has a prominent protective effect against liver aging. For liver tissues, it is demonstrated that impaired autophagy contributes to aging [35]. Interestingly, here in the study coconcomitant with accumulation of defective mitochondria, autophagy declined and more intracellular lipid droplets were found in the α-gal group. α-gal + Rg1 group regained autophagy showing autophagosomes, however compared with the control group, more telosomosomes including lipofuscin and myelinfigure were found in the hepatocytes, which are the biomarker of declining autophagy representing undigested material inside hepatocytes (Fig. 2D).

Apoptosis progression was further analyzed. Limited apoptotic cells were displayed in histological sections with both H&E routine staining and TUNEL staining (Fig. 3A, B). The results of Western Blot showed that pro-apoptotic proteins Bax and Bim increased in the liver treated with α-galactose, whereas Rg1 decreased the level of pro-apoptotic proteins. However, this does not mean α-galactose promoted apoptosis, as anti-apoptotic protein Bcl-2 concurrently increased significantly in the α-gal group. Finally, there was no statistical significance of Bax/Bcl-2 ratio between the α-gal group and the control group according to the semi-quantitative analysis of these two proteins, suggesting apoptosis was resisted during α-galactose treatment (Fig. 3C–E). In summary, α-galactose treatment mainly caused senescence progression not apoptosis to livers.

3.3. Rg1 protected against the α-galactose induced fatty liver disease

Histopathological examinations of H&E stained liver sections of α-gal group showed obvious hydropic degeneration in hepatocytes around portal area. Micro-vascular hepatocellular vacuolation, nuclear chromosome aggregation concurrent with swelling of mitochondria indicated cell membrane energy-dependent ion pump dysfunction after α-galactose treatment. However, Rg1 repaired the degeneration to a large extent (Fig. 4A). Besides, lymphocytes infiltration was found in the α-galactose treated hepatic lobe, whereas Rg1 inhibited inflammation (Fig. 4A). Furthermore, hepatocellular metabolic function was evaluated. In the α-gal group, Oil Red O stain revealed numerous small red lip droplets within the hepatocytes near the central vein demonstrating significant fat deposition in the liver; however, after Rg1 treatment fat accumulation was attenuated (Fig. 4B). Hepatic glycogen reflects the metabolic balance between glucose and glycogen. Liver sections of the control and Rg1 group demonstrated cytoplasmic purple-magenta colored PAS positive hepatocytes. In contrast, the α-gal group showed marked glycogen depletion in cytoplasm of hepatocytes, however Rg1 replenished hepatic glycogen storage (Fig. 4C). As senescence-associated secretory phenotype (SASP) which can induce inflammation and attract immune cells, proinflammatory cytokines including IL-1, IL-6 and MCP-1 were detected. Compared with the control group, increases in IL-1, IL-6 and MCP-1 were found in α-galactose treated liver, whereas decreases were revealed in Rg1 singly treated livers. Also, Rg1 post-treatment restored the level of IL-1, IL-6 and MCP-1 in the α-galactose treated livers (Fig. 4D–F). All these data above suggested α-galactose treatment induced hepatic steatosis accompanied by other metabolic dysregulation and inflammatory phenomena, interestingly Rg1 protected liver against α-galactose induced NAFLD.

3.4. Rg1 enhanced liver FOXO1

To elucidate the question that α-galactose induced senescence-related fatty liver disease and the protective effects of Rg1 is related to FOXOs, we assessed the phosphorylation and protein levels of FOXO1. The results of Western Blot and Immunohistochemistry are of the same trend (Fig. 5A–C). Compared with the control group FOXO1 expression in hepatocytes of α-galactose treated group dropped dramatically concurrent with increased p-FOXO1 expression, causing degradation of FOXO1. However, no matter single treatment without α-galactose or post-treatment with α-galactose, Rg1 significantly elevated FOXO1 in hepatocytes. Followed that decrease in FOXO1 targeted antioxidant enzymes SOD and CAT was found in the α-gal group whereas elevation of SOD and CAT was demonstrated in the α-gal + Rg1 group; on contrary increase of malondialdehyde (MDA), indicator of lipid peroxidation, increased in the α-gal group whereas Rg1 diminished MDA level (Fig. 5D–F). These results indicate that FOXO1 may be involved in α-galactose induced hepatic aging, steatosis, glucose and lipid metabolic dysregulation. Rg1 plays a role in FOXO1 up-regulation to enhance cellular anti-oxidative potential meanwhile to alleviate lipid peroxidation, also to keep metabolic homeostasis, which may be the mechanism for protection livers from senescence and NAFLD.

4. Discussion

In response to various oxidative stresses such as exposure to oxidants, γ-irradiation and DNA damaging chemotherapies, cells may undergo apoptosis which is a form of programmed cell death or a persistent proliferative arrest known as cellular senescence. Both of them are cellular protective mechanisms for oxidative stress, however, apoptosis may remove damaged or pre-neoplastic cells, indicating a more powerful potential of restricting tumorigenesis than senescence [36,37]. In contrast, senescent cells are stable viable and may influence neighboring cells via secreting SASP [38,39]. Therefore, senescent cells accumulate to an organism’s detriment even tumor-promoting effects in adults. Senescence as a fundamental cellular process is closely related to procession of chronic liver diseases including NAFLD, suggesting it should have great diagnostic, prognostic and therapeutic potential in NAFLD. In the present study, we provide the first evidence that Ginseng extract, Rg1, attenuated senescence-associated liver damage in a α-galactose induced senescence mouse model. Most importantly, Rg1 treatment significantly up-regulated FOXO1 and alleviated hepatic oxidative stress and restored hepatic function, reversed liver pathological changes, including hydropic degeneration, lipid accumulation, hepatic glucose decrease, elevated SASP and inflammation. The current study revealed that anti-aging property and role in FOXO1 regulation might be the key mechanisms of Rg1 to fight against NAFLD.

Amounts of evidences show that the pathogenesis of NAFLD involves two-hit process. The first hit is the steatosis which is believed to
be triggered by insulin resistance [40]. Although oxidative stress which results in disease progression is the key factor in the second hit, whether oxidative stress senescence itself induced can cause hepatic damage and the definite mechanisms remain debatable. Here, 42-day-treated D-galactose was used to mimic generated reactive oxygen species (ROS) in the chronic diseased liver. It was found that after D-galactose treatment senescence biomarker senescence-associated β-galactosidase (SA-β-Gal), P53, P21 proteins were significantly elevated in hepatocytes compared with the control group, mitochondrial dysfunction was also presented. However, hepatocytes seemed resistant to apoptosis as indicators of apoptosis were seldom increased. These data were in line with the results got from another group before. The inverse relationship

**Fig. 3.** No obvious apoptosis progression occurred via D-galactose treatment. (A) Liver paraffin sections were stained using H&E and imaged at a magnification of 400×. In the D-gal-treated group, scanty hepatocyte apoptosis occurred as showing densely and deeply stained nuclear chromatin and enhanced eosinophilic staining of cytoplasm. (B) Paraffin sections of liver tissue were stained with TUNEL reagent (magnification 400×). Few positive cells occurred in the D-galactose treated group. (C) Western blots were performed to determine the expression level of pro-apoptosis and anti-apoptosis related proteins. (D, E) A histogram of relative protein expression is presented. Data were presented as means ± SD (n = 5/group). **P < 0.01; ***P < 0.001.
between P21 and apoptosis sensitivity was reported. In apoptosis P21 is actively suppressed, and senescence is partially replaced by compensatory apoptosis in P21-knockout animals. In addition, Bcl-2 family proteins which manipulate upstream mediators of apoptosis can influence the alternative fates between senescence and apoptosis [41].

Evidences have shown that over-expression of Bcl-2 forced senescence in fibroblasts treated with lethal dose of doxorubicin, also over-expression of Bcl-2 caused P53-dependent senescence rather than apoptosis in cyclophosphamide-treated murine lymphomas [42]. In the current study, d-galactose treatment triggered an increase of Bcl-2, P53 and its downstream P21, which drove hepatocytes towards senescence rather than apoptosis. Therefore, senescence model of the liver was successfully set up by using d-galactose to mimic the clinical scenario in chronic liver disease.

Notably, the more recently described functions of oxidative stress-suppressed autophagy are highly relevant to the pathogenesis of NAFLD including the ability of macroautophagy to regulate cellular insulin sensitivity, metabolize cellular lipid stores, mediate hepatocyte resistance to injurious stimuli such as oxidants and cytokines. The relationship between autophagy decline and hallmarks of aging has been carefully studied in livers. Documents reported aged liver tissues have a diminished activity of autophagy. Lipofusin is the oldest and simplest biomarker of declining autophagy, also in aged livers of humans and mice, levels of autophagy-related genes and their proteins Atg7, Atg5, Atg4B, Beclin-1 and Lamp-2 are dramatically reduced. Amounting of evidences demonstrated impaired autophagic function contributes to hepatocyte aging and potential mechanisms are probably related to energetic comprise of the aging cells, negative regulation by insulin receptor, upregulation of insulin/IGF-1 and activated mTOR, decreased turnover of mitochondria, fusion deficiency between autophagosomes and lysosomes, weak function of lysosome enzymes [35,43]. Bcl-2 proteins not only counteract the activity of proapoptotic proteins to downregulate apoptosis, but interact with Beclin-1 to impede autophagy as well. Autophagy can also precede apoptosis through caspase-
Fig. 5. Rg1 ameliorates the decrease in liver FOXO1 expression induced by α-galactose treatment. (A) Western blots were performed to determine the protein expression level of FOXO1 and p-FOXO1 in the liver. β-actin was probed as a loading control. (B) A histogram of relative protein expression is presented. *P < 0.05; **P < 0.01; ***P < .001. (C) FOXO1 expression in liver histological section detected by immunohistochemistry (magnification 200×). (D) A semi-quantitative analysis of the ratio of FOXO1 positive staining to the total field. (E-G) Rg1 reverses the level of oxidative indices and FOXO1 targeted antioxidative enzyme examined by enzymatic assay.
The crucial role of cellular senescence in NAFLD progression makes it an ideal target for disease therapy. Treatment targeted towards eliminating senescent hepatocytes should, in theory, help alleviate NAFLD [53,54]. However, drugs which target whether anti-apoptotic protein BCL-2 or regulation of SASP seldom undergo genuine applicability so far [53]. Ginseng is widely used Chinese traditional medicine. In > 30 types of ginsenoside, Rg1 is regarded as the main active ingredient responsible for pharmacological effects with multi-target property [55]. Previous studies have confirmed that Ginsenoside Rg1 play a role in anti-aging, anti-oxidation, hematopoiesis promotion, immunity improvement and neuron growth [56,57]. Different targeted pathways were found which involved in Ginsenoside Rg1 function. Ginsenoside Rg1 may exert an antioxidant role by regulating Wnt/β-catenin pathway and Nr2f-Keap1 signaling pathways [58,59]. Ginsenoside Rg1 plays an anti-aging role by regulating p19-p53-p21pathway, Wnt/β-catenin pathway and Nr2f-Keap1 signaling pathways to ameliorate oxidative stress [60,61]. Rg1 may effectively regulate inflammatory response by inhibiting the activation of the NF-kB signaling pathway [62]. Nr2f signaling pathway, PPAR signaling pathway, MAPK signaling pathway, and Toll-like receptor signaling pathway may be intervened by Rg1 and targeted on cardiomyocytes, neurons, and so on, protect against arthritis, glomerular nephritis and acute liver injury [63-66]. Rg1can also exert protective roles via regulating autophagy related genes including mTOR, LC3-II, P62, Beclin-1 and Bcl-2 to enhance autophagy to protect cardiomyocytes, macrophages, neurons, and kidney podocytes [67,68]. As regard as liver diseases, researches spotlighted on hepatoprotective effects of Rg1 via reduction in oxidative stress, anti-inflammation, and anti-fibrosis [69,70]. Here this study indicates that restoration of liver function via pharmacological elimination of senescent cells is now a real possibility. Ginsenoside Rg1 protected hepatocytes from α-galactose-induced senescence and ameliorated oxidative burden and inflammation in hepatic microenvironment. Most importantly, it is first evidenced that Rg1 maintained FOXO1 activity in hepatocytes, hence Rg1 protected the liver from NAFLD. Taken that Ginseng is well tolerated and the side effects are mild and reversible [71,72], the clinical therapeutic use of Ginsenoside Rg1 to disorders of chronic hepatotoxicity, hepatitis, hepatic fibrosis and cirrhosis would be promising.

5. Conclusions
The results of this study indicate that Rg1 may attenuate senescence-associated liver damage. Rg1 treatment can restore hepatic function and maintain homeostasis of hepatic glucose production and lipometabolism, the mechanism of which may be involved in up-regulating FOXO1 and alleviating hepatic oxidative stress. The anti-aging property of Rg1 and its role in FOXO1 regulation provide a possible target for the treatment of NAFLD. However, further experiments are needed to investigate the mechanisms that Rg1 protects against liver damage.

**Abbreviations**
- Rg1: α-galactose
- ROS: Reactive oxygen species
- ALT: Alanine aminotransferase
- AST: Aspartate aminotransferase
- SOD: Superoxide dismutase
- CAT: Catalase
- MDA: Malondialdehyde
Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Author contributions

For research articles, Lu Wang and Yaping Wang conceptualized and designed the experiments; Rongjia Qi and Rong Jiang performed the experiments; Hanxianzi Xiao, Ziling Wang and Siyuan He contributed reagents/materials/analysis tools; Rongjia Qi analyzed the data and designed the experiments; Rongjia Qi and Rong Jiang performed the experiments.

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